REMARKS

In the interest of furthering prosecution, Claim 1 has been amended and Claims 4, 9, and 11-13 have been cancelled, without prejudice or disclaimer, and without acquiescing to the rejections. Support for the amendment of Claim 1 can be found in Claim 4 as originally filed.

New Claims 14 - 25 have been submitted. Support for new Claim 14 can be found in paragraphs [0019], [0047], [0048], [0057], [0058] and [0066] of the Applicants' Specification and originally filed Claims 11 and 13. Support for Claims 15-24 can be found in Claims 1-13 as originally filed.

Claim Rejections under 35 U.S.C. § 112, first paragraph

Claim 12 stands rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the enablement requirement. Claim 12 has been cancelled solely for the purpose of expediting allowance. Thus, the rejection of Claim 12 is moot.

Claim 8 stands rejected under 35 U.S.C. § 112, first paragraph as allegedly containing subject matter that was not adequately described in the Specification. Specifically, the Official Action alleges that Claim 8 is drawn to the inclusion of agents in addition to the oil of *Helichrysum italicum* that augment the production of collagen and VEGF, but there are no teachings as to other agents that could be further added to the composition.

The Applicants respectfully note that Claim 9, and not Claim 8, concerns the further addition of an agent that augments the production of collagen and VEGF. Claim 8 recites the further addition of a skin compatible carrier. Such carriers are well known in the art. This language is also fully supported by the Applicants' Specification. Accordingly, the Applicants request removal of the rejection of Claim 8.

Furthermore, in the interest of expediting prosecution, Claim 9 has been cancelled without prejudice or disclaimer. Thus, the basis of the rejection is moot.

Claim Rejections under 35 U.S.C. § 112, second paragraph

Claims 1-13 stand rejected under 35 U.S.C. § 112, second paragraph as allegedly failing to distinctly point out and claim the subject matter regarded as the invention. The Official Action specifically cites the term "substantially" in Claim 1 as indefinite. Claims 2-13 are believed to be rejected due to dependence on Claim 1.

Claim 1 has been amended to delete the term "substantially" solely for the purpose of expediting allowance. Accordingly, the rejection based on the recitation of this term is moot and removal is respectfully requested.

Additionally, Claim 8 stands rejected under 35 U.S.C. § 112, second paragraph for essentially the same reasons discussed with respect to the rejection under 35 U.S.C. § 112, first paragraph. As discussed above, Claim 8 does not describe the further addition of an agent that augments the production of collagen and VEGF, and removal of the rejection is respectfully requested.

Claim Rejections under 35 U.S.C. § 102(b)

Claims 1- 4, 8, and 11-13 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Amrita ("*Helichrysum italicum,*" 1999, XP002224497). Specifically, the Official Action alleges that Amrita teaches the skin rejuvenating properties of an essential oil extracted from the flower tops of *Helichrysum italicum,* which would also inherently possess the same composition and properties as the subject matter of the rejected claims.

The Applicants respectfully submit that the essential oil disclosed in Amrita is not identical or substantially identical to the essential oil recited in the rejected claims. For example, the preparation of an essential oil of *Helichrysum italicum* described in the prior art comprise only 28.9% neryl acetate or less (See paragraph [0004] of the Applicants' Specification). Indeed, a recent study that analyzed the composition of the flowers of *Helichrysum italicum* determined that the flower oil possesses between 16.6% and 26.6% neryl acetate (See Table 3 of Angioni et al. (2003) "Chemical Composition, Plant Genetic Differences, and Antifungal Activity of the Essential Oil of *Helichrysum italicum* G. Don ssp. *microphyllum* (Willd) Nym.," *J. Agric. Food Chem.* 51(4): 1030-34; copy enclosed).

In sharp contrast, the Applicants point to paragraph [0021] of the Applicants' Specification, which recites:

> The composition of the essential oil of *Helichrysum italicum* from Corsica is very original. Its principal constituent, neryl acetate, is present in a very high proportion, from about 40 to about 50% on average, and up to about 70% in the Balagne mountains in the northwestern part of Corsica.

Due to the lack of adequate teaching in the art of *Helichrysum italicum* oil with such an unusually high concentration of neryl acetate, one skilled in the art would not expect that known *Helichrysum italicum* essential oils possess 40-70% neryl acetate. Indeed, as there is no evidence on this record to the contrary, one skilled in the art would expect that the essential oil of *Helichrysum italicum* known in the art, including Amrita, possesses no more than 28.9% neryl acetate. Thus, Claim 1 embodies a unique concentration range of neryl acetate and is distinct from Amrita.

Furthermore, the Applicants' Specification teaches that the high percentage of neryl acetate provides the essential oil with such particular properties as sixfold multiplication of the production of collagen I and augmentation of the production of VEGF on the order of twofold (See paragraphs [0017]-[0024] of the Applicants' Specification). As one skilled in the art would not reasonably expect that the oil disclosed in Amrita possesses this high concentration of neryl acetate, they would also not expect it to possess the same properties and activities as an oil comprised of 40-70% neryl acetate. Therefore, in addition to being compositionally distinct, the claimed composition and Amrita's oil are also functionally distinct.

Based on the foregoing, the Applicants respectfully submit that Amrita does not anticipate the composition described in Claim 1 because the Amrita's essential oil is neither compositionally identical nor is it reasonably expected to possess the same properties. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(b) over Amrita is respectfully requested.

Claims 1-4, 8, 11-13 stand rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Tyler (US 5,785,972). The Official Action alleges that Tyler discloses a composition comprising the oil of *Helichrysum italicum* for topical application and, thus, anticipates the rejected claims.

As discussed with respect to the rejections over Amrita, one skilled in the art would expect that the essential oil of *Helichrysum italicum* possesses no more than 28.9% neryl acetate. In sharp contrast, Claim 1 recites a significantly higher concentration of neryl acetate, which confers the properties and activities relevant to treatment of skin aging. As Tyler does not explicitly teach an essential oil with the high neryl acetate concentration, one skilled in the art would reasonably expect that the oil has a concentration less than 28.9% and, thus, lacks the properties of the composition recited in Claim 1. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(b) over Tyler is respectfully requested.

Claims 1-5 and 8 stand rejected under 35 U.S.C. § 102(b) over Spina (Derwent Acc. No. 1999-471299) as evidenced by Tyler. The Official Action alleges that Spina discloses a composition comprising *Helichrysum italicum* for topical application and, thus, anticipates the composition described in the rejected claims. Tyler is relied upon as allegedly suggesting that the composition of Spina specifically comprises the essential oil of the flower of *Helichrysum italicum.*

As discussed above with respect to Amrita, one skilled in the art would expect that the essential oil *Helichrysum italicum* in both Spina and Tyler possesses no more than 28.9% neryl acetate, and not a neryl acetate concentration of 40-70%. Furthermore, as this high concentration of neryl acetate confers the properties and activities relevant to treating skin aging, one skilled in the art would not reasonably expect that an oil with a concentration less than 28.9% would have the same properties as the composition recited in Claim 1.

Due to the fact that neither the provided translation of Spina nor Tyler explicitly teaches an essential oil expected to have a 40-70% neryl acetate concentration, the hypothetical combination of these documents does not teach the composition recited in Claim 1. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(b) over Spina in view of Tyler is respectfully requested.

Claim Rejections under 35 U.S.C. § 103(a)

Claim 6 stands rejected under 35 U.S.C. § 103(a) as allegedly obvious over Spina in view of Tyler for the same reasons set forth in the rejection of claims 1-5 and 8 under 35 U.S.C. § 102(b). Furthermore, the Official Action alleges that Claim 6 recites a specific amount of oil in relation to the composition that amounts to routine optimization and is, thus, an allegedly obvious variation.

The Applicants respectfully submit that one skilled in the art would expect that the essential oil of *Helichrysum italicum* described in both Tyler and Spina possesses less than 28.9% neryl acetate and, thus, lacks anti-wrinkle properties. Moreover, neither Spina nor Tyler provide motivation to optimize the concentration of neryl acetate in the oil or how to do so. Moreover, there is nothing in either publication that indicates that there would be any advantage to increasing the amount by more than a third to the claimed 40-70% neryl acetate, as recited in Claim 1.

Regional and seasonal difference can also produce significant variation in neryl acetate concentration. For example, essential oil obtained from plants native the Balagne mountains in the northwestern part of Corsica possess 70% neryl acetate concentrations, whereas plants from other parts of Corsica have only 40-50%. Angioni et al. describes oil obtained from plants in Cagliari, Italy which possess 26.6% neryl acetate in June, 23.1% in July, and 16.6% in September. Therefore, even if one skilled in the art was hypothetically motivated to make an anti-wrinkle composition using oil with 40-70% neryl acetate and in view of Spina and Tyler, such a person would still have no reasonable expectation of obtaining oil with the claimed concentrations.

Based on the foregoing, the Applicants respectfully submit that one skilled in the art in view of Spina and Tyler would neither be motivated or enabled to make the composition recited in Claim 1. Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 103(a) over Spina in view of Tyler is respectfully requested.

Claims 7 and 8 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over Amrita in further view of Afirat et al. (US2002/0119954). Specifically, the Official Action alleges that Amrita teaches one skilled in the art to make and use the claimed composition and Afirat et al. makes it obvious to further incorporate the composition into nanospheres.

The Applicants respectfully submit that one skilled in the art would expect that the essential oil of *Helichrysum italicum* described in Amrita possesses less than 40% neryl acetate and, thus, lacks anti-wrinkle properties. Afriat et al., which provides no teaching specifically pertaining to the essential oil of *Helichrysum italicum,* fails to cure this deficiency. Accordingly, the Applicants submit that the claims are not anticipated, and reconsideration and withdrawal of the rejections under 35 U.S.C. § 103(a) over Amrita in view of Afriat et al. is respectfully requested.

In light of the foregoing, the Applicants respectfully submit that the entire Application is now in condition for allowance, which is respectfully requested.

Respectfully submitted,

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Chemical Composition, Plant Genetic Differences, and Antifungal Activity of the Essential Oil of *Helichrysum italicum* G. Don ssp. *microphyllum* (Willd) Nym

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The chemical composition of the essential oil of the Sardinian dwarf curry plant [*Helichrysum italicum* G. Don ssp. *microphyllum* (Willd) Nym] was studied. Genetic analysis suggested the presence of two chemotypes; morphological and chemical differences confirmed the presence of two chemotypes (A and B). The maximum yields were 0.18 and 0.04% (v/w) for flowering tops and stems, respectively. The concentrations of nerol and its esters (acetate and propionate), limonene, and linalool reach their highest values during the flowering stage both in flowers and in stems. Besides the essential oil, type B showed an interesting antifungal activity.

KEYWORDS: ***Helichrysum italicum* ssp. *microphyllum*; essential oil composition; ontogenetic variation; antifungal activity**

INTRODUCTION

Helichrysum is a typical aromatic plant of the Asteraceae family. There are ~300 species of *Helichrysum* (*1*), 16 of which are spontaneous in Europe (*2*). Among the large number of phytoproducts that can be obtained from *Helichrysum*, the essential oil has an important role. It is used both in cosmetics and in pharmaceutical preparations (*2–9*). Studies carried out on the chemical composition of the essential oils from different species of *Helichrysum* (*H. amorginum*, *H. italicum*, *H. serotinum*, *H. stoechas*, and *H. taenari*) showed the same constituents but in different concentrations (*1–9*). In a recent paper reporting on two different chemical compositions of the essential oil of *H. italicum* ssp. *microphyllum*, Satta et al. (*10*) proposed the presence of two different chemotypes, one rich in nerol and its esters (~50%), the other rich in *ar*-curcumene, γ-curcumene, and rosifoliol (*11*). This distinct difference has led to the hypothesis that this plants are not only ecotypes but different chemotypes.

Natural plant species are characterized by a high degree of intervariety polymorphism; one method to assess the polymorphism degree is the use of the random amplified polymorphic DNA (RAPD) technique, coupled with principal component analysis (PCA) (*12*, *13*). The RAPD technique involves the amplification of genomic DNA using short primers, called "random primers". When this approach is used, different electrophoretic patterns can be obtained from the mutation of a single basis, so the genetic pathway reveals molecular polymorphism between DNA samples from different organisms.

As is well-known, the use of PCA allows a more objective interpretation of the RAPD fingerprint, reducing the descriptor parameters to two or three only (*14*).

Usually, the oil is obtained by distillation of fresh flowering tops, harvested during the flowering stage. Neither the effect of the different parts of the plant nor that of the harvesting period has been reported in the literature. Moreover, it is known that essential oils could be used as biopesticides (*15–17*); no paper was found for *Helichrysum* essential oils in the literature. The aims of the present paper were (a) to assess the genetic differences between the two ecotypes of *Helichrysum*, (b) to study the fungi toxic activity against plant pests of the two different oils, and (c) to find the best harvesting period and to assess the contribution of the different plant parts (flowers/stems) to the yield in essential oil of *Helichrysum italicum* ssp. *microphyllum* and to its composition.

EXPERIMENTAL PROCEDURES

(a) Genetic Analysis. *Plant Material.* DNA samples were obtained from leaves and flowers collected from wild plants of the two types A

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and B (five samples each) and from plants grown at the same altitudes (40 m, slm) in an experimental field in Uta (Cagliari, Italy), from seeds of *Helichrysum* collected in the original areas. After 3 years, eight samples of type A and four samples of type B were collected. The morphological characteristics, between the two types of plant, remained unchanged during the 3 year trial, corresponding to the characteristics of the original plants: type A, stems were intensely green and tender and the flowers agreeably perfumed; type B, stems were slightly green-gray and woody and the flowers only slightly perfumed.

DNA Reagents and Instrumentation. dNTPs DNA ladder 100 bp used for RAPD-PCR analysis were from Promega (Milan, Italy Promega, S.r.l.), and Taq-polymerase, $MgCl_2$, and tampone 10× were from Qiagen (Milan, Italy). Primers were synthesized by Amersham-Pharmacia Biotech. To obtain pure DNA, 100 mg of *Helichrysum* was extracted with Nucleon Phytopure plant and fungal DNA extraction kit (Amersham-Pharmacia Biotech), specific for vegetable material. The extracted DNA was dissolved in 200 μL of TE 1× buffer (0.01M Tris-HCl, 1 mM EDTA-Na_2).

Amplifications were carried out using a PCR system 9700 (PE Applied Biosystems). The electrophoretic analysis was carried out with a Power Pack 300 power supply, a subcell agarose gel electrophoresis system (Bio-Rad) generator, for gel electrophoresis. Gels (at 2%) were prepared with agarose LE from Euroclone.

The DNA was stained using ethidium bromide; all RAPD profiles were analyzed using a Fluor-S MultiImager detector, equipped with Quantity One software from Bio-Rad (Segrate Milan, Italy).

DNA Amplification and Detection. The reaction medium was prepared by mixing 11.9 μL of H_2O twice distilled, 2.5 μL of 10× buffer [100 mM Tris-HCl, 500 mM KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$, pH 8.7, a 20 °C], 2 μL of dNTPs (dATP, dCTP, dGTP, and dTTP, 2.5 mM cad.), 1 μL of 10 μM oligonucleotide primer, 2.5 μL of 25 mM $MgCl_2$, 0.5 unit of Taq polymerase, and 5 μL of genomic DNA (20 ng/μL). Fifty microliters of mineral oil was added at the final volume of 25 μL. Eppendorf tubes of 500 μL were placed in the thermocycler with the following program: initial denaturation at 95 °C for 7 min, 45 amplification cycles at 95 °C for 30 s, 35 °C for 30 s, 72 °C for 40 s, and a final step of extension at 72 °C for 7 min. Eleven primer RAPDs (operon) were used to amplify the 18 samples of *Helichrysum*: A1, CAGGCCCTTC; A2, TGCCGAGCTG; A3, AGTCAGCCAC; A4, AATCGGGCTG; A5, AGGGGTCTTG; A7, GAAACGGGTG; A8, GAAACGGGTG; A12, TCGGCGATAG; A13, CAGCACCCAC; A14, TCTGTGCTGG; A15, TTCCGAACCC. Aliquots of 15 μL of amplified sample were loaded in agar gels at 2% and analyzed by electrophoresis for 20 min at 60 V and for 90 min at 90 V, in TBE buffer 0.5× (8.8 mM Tris-HCl, 8.8 mM boric acid, and 0.2 mM EDTA). Identification of the strips was carried out using ethidium bromide and computerized analysis of the image.

(b) Microbiological Assay. *Paper Test.* The effectiveness of the essential oils of *Helichrysum* on 11 plant fungi was evaluated. The fungi were *Botrytis cinerea, Cercospora beticola, Fusarium oxysporum lycopersici, Fusarium graminearum, Helminthosporium oryzae, Pythium ultimum, Pyricularia orizae, Rhizoctonia solani, Sclerotium rolfsii, Phytophthora capsici*, and *Septoria tritici*.

Paper dishes (12.7 mm diameter) were soaked in a solution of essential oil/acetone (1/1) and stored for several days at 24 °C in Petri dishes (30 mm diameter) containing agar potato dextrose and fungus mycelia. All of the experiments were performed in triplicate. Inhibition of fungal growth (dependent on the rate of fungal growth) for each test fungus was measured after treatment. Sensitivity of the fungal species to the oils was determined by comparing the sizes of inhibitory zones (*19*). Mycelium growth was classified with (−) when mycelia exceeded the paper border, (+−) when mycelia had a slow initial growth but later exceeded the paper border, and (+) when mycelia do not grow.

Fungicide Activity (MIC). To determine the MIC, a stock solution of the essential oil (1 mg/mL) was prepared in a sterile broth at 50% of EC oil (ethanol chemical).

Working standard solutions at different diluting factors were prepared from the stock solution by dilution with 100 μL of sterile potato dextrose broth (PDB) medium in a 96-hole multiwell, together with 1 μL of fungal suspension (~1 × 10^5 cells) in each well.

During the experiments the growth conditions and the medium sterility were checked for each strain. The incubation conditions were the same as those in the paper test.

The efficacy of the essential oils was compared to that of synthetic fungicides commonly used in agriculture treatments (benomyl, tetraconazole, metalaxyl, chlozolinate, and kresoxym-methyl).

(c) Harvest: Period and Method. *Plant Material.* Samples of type A were collected from a natural *Helichrysum* field of ~1 ha, with a plant density of 0.5 plant/m^2, at Esterzili (Cagliari, Italy) at an altitude of 1200 m above sea level. The field was divided into four randomized blocks with three replications; each block was composed of ~125 plants. All of the plants were sampled, and the fresh flowering tops and stems (10 cm) were collected separately. The samples were carried in jute bags at 22 °C. Samples of ~4 kg were collected for each replicate, corresponding to ~30 g per plant. The plants were harvested before (June 5), during (July 10), and after the flowering stage (September 19). The specimens were identified and deposited in the Herbarium CAG of the Department of Botanical Sciences of the University of Cagliari.

Distillation. Homogeneous samples of flowering top and stems were distilled separately. The different plant portions (130 g) were steam distilled for 1 h in a Clevenger-type apparatus according to the Italian Official Pharmacopoea X (*18*). Three replicate samples were distilled simultaneously. The essential oil was recovered directly using a micropipet from above the distillate without adding any solvent.

Chemicals. Linalool, limonene (Extrasynthese, Genay, France), α-terpineol, nerol, neryl acetate, and guaiol (Aldrich, Acros, and Fluka, Milan Italy) were used as analytical standards (≥97%). 2,6-Dimethylphenol was used as an internal standard (99.8%; Aldrich, Milan, Italy). Solutions of 1% (w/v) oil were prepared in hexane for GC (Carlo Erba, Milan, Italy).

GC/MS Analysis. A Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Avondale, PA), equipped with an MS detector HP 5971 A, an HP 7673 autosampler, a split−splitless injector, and an MS ChemStation HP v. C.00.07, was used. The column was a fused silica capillary DB-5MS (5% phenylmethylpolysiloxane, 30 m × 0.25 mm; film thickness = 0.25 μm) (J&W Scientific Fisons, Folsom, CA). The injector and interface were operated at 200 and 280 °C, respectively. The oven temperature was programmed as follows: from 60 to 180 °C (3 °C/min) and isothermally held for 15 min. Helium was the carrier gas at 0.9 mL/min; the sample (1 μL) was injected in the split mode (1:20). MS conditions were as follows: ionization voltage, 70 eV; scan rate, 1.6 scan/s; mass range, 40−500; ion source temperature, 180 °C. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention index (LRI) relative to the series of *n*-hydrocarbons, and on computer maching against commercial (NIST 98 and ADAMS) and homemade library mass spectra built up from pure substances and components of known oils and MS literature data.

Quantitative Analysis and Data Expression. The data from the GC-MS were acquired in SIM mode. The analyses were based on the following ions: limonene, m/z 68; linalool, m/z 71; α-terpineol, m/z 59; nerol, neryl acetate, and neryl propionate, m/z 69; *ar*-curcumene and γ-curcumene, m/z 119; guaiol, m/z 161; rosifoliol, m/z 149; 2,6-dimethylphenol (i.s.), m/z 122. The contents of components in each essential oil were calculated from GC-MS areas related to GC-MS areas of the internal standard. Percentage of total was obtained by their addition.

Statistical Analysis. Analysis of variance ANOVA was performed by using "Statistics for Windows", when appropriate ($p < 0.05$); analysis was followed by the Tukey post hoc test.

PCA was performed using Mathematica software.

RESULTS AND DISCUSSION

The oil yields were 0.16 ± 0.06 (%, v/w). The essential oils, obtained from the plants of *Helichrysum* cultivated at the same altitudes, have shown strong differences in their physicochemical characteristics, in accord with those of the original areas. In fact, the oils of type A are clear, light yellow, and liquid at

Table 1. Fungicidal Activity on Paper Test of the Essential Oils

oil	fungus										
	BOTCI	CERBE	FUSXL	FUSGR	HELOR	PYTUL	PYROR	SCLRO	RHISO	PHYCA	SEPTR
type A	+	+−	+	+	+	+	+	+−	+	+	+
type B	+	+	+	+	+	+	+	+	+	+	+

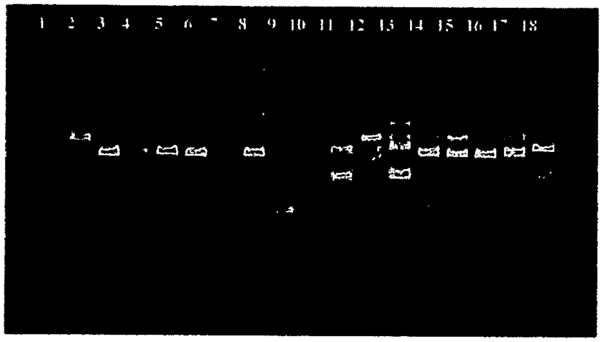

Figure 1. Example of RAPD fingerprint obtained using A7 primer: lanes 1–4, *Helichrysum* type B cultivated; lanes 5–9, *Helichrysum* type B wild; lanes 10–13, *Helichrysum* type A cultivated; lanes 14–18, *Helichrysum* type A wild.

both 4 and 25 °C, whereas those of type B are cloudy, thick at 25 °C, and of a semisolid waxy consistency at 4 °C.

(a) Genetic Analysis. *PCA Analysis.* Genomic DNA extracted from *Helichrysum* samples was amplified using the RAPD protocol.

To study *Helichrysum* polymorphisms, we first amplified DNA using nine random primers. All profiles showed a typical complex RAPD pattern, up to eight principal bands (**Figure 1** shows a typical result of RAPD amplification). RAPD profiles revealed the appearance of several amplicons, some of which show a good intensity after ethidium bromide labeling. Each profile was then analyzed by means of the count of the bands the "classic" approach to establish the polymorphism degree is obtained, considering the number of common bands toward total band number generated by means of RAPD amplification. In this case, we have arbitrarily assigned some values to all bands generated. An arbitrary value of 1 was established to indicate the presence of a band in a lane; a value of 0 represents the absence of the corresponding band in other lanes (different samples), and variable value from 0 to 1 (value based on the total mean bands) indicates the "not amplified" lanes. In fact, this value indicates the probability of amplifying of a single band, even if no band is visible in the corresponding lane. The information obtained by RAPD analysis was transferred in a graph, employing PCA analysis. **Figure 2** show the clustering obtained by PCA using data from nine random primers. The samples were identified in two different areas of the plane (PC1 = type B; PC2 = type A). Samples numbered 5, 7, and 10 give unexpected results, although with all of the primers used, so it can be assumed that a genetic difference between *Helichrysum* type A and type B could exist.

Therefore, we have increased the number of random primers to generate more fingerprints to describe the *Helichrysum* samples. The use of more primers (11) did not improve the clustering of these samples. The medium score of these statistical analyses did not show any significant improvement, changing from 66.52% (9 primers) to 65.65% (11 primers).

Chemical Analysis. The total ion current (TIC) chromatograms confirmed the qualitative differences present in the oils. The identified compounds represent almost 90% of the essential

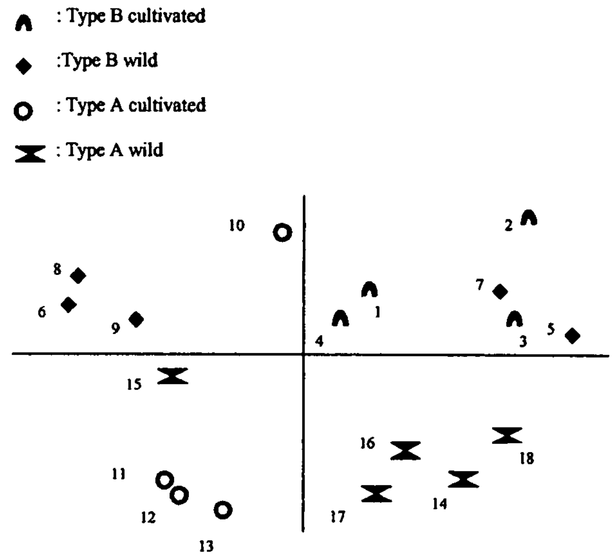

Figure 2. Graph obtained translating the acquired information using the RAPD technique with PCA.

oil. Nerol and its esters (acetate and propionate), which are the main components of oil type A oil, were completely absent in the type B oil, which was rich in rosifoliol. No qualitative difference was found between the wild and cultivated essential oils; these data are in accord with those of Satta et al. (*10*).

(b) Microbiological Analysis. Table 1 reports the data from the paper test. Both types of *Helichrysum* samples showed inhibition against fungal growth, except *Helichrysum* type A for *Cercospora beticola* and *Pyricularia oryzae*. For these two fungi an initial inhibition followed by a remarkable growth was observed. The MIC data reported in **Table 2** showed a good action of *Helichrysum* type B on *Pythium ultimum* and *Sclerotium rolfsii* and a moderate action against *Phytophthora capsici* and *Septoria tritici*. *Helichrysum* type A has shown a much lower antifungal activity.

(c) Harvest: Period and Method. The flowers and stems of *H. italicum* ssp. *microphyllum* type A were weighed after every harvest. The flower/stem ratio was calculated; it was 1/1 for the first period, 2/1 for the second period, and 3/1 for the third period. This means that the stem growth was not proportional to the flower growth.

The yields in oil (v/p) (fresh weight) from the flowers were 0.11 ± 0.02% in June, 0.13 ± 0.03% in July, and 0.18 ± 0.04% in September, whereas those from the stems were 0.03 ± 0.006% in June, 0.03 ± 0.002% in July, and 0.04 ± 0.002% in September. The highest yield in oil coincided with the end of the flowering stage, even though it was not statistically different from that of the flowering stage.

Table 3 shows major compounds (%; w/w) of the essential oil of *H. italicum* ssp. *microphyllum* in the flowers and stems of type A in the three harvesting periods.

The reported compounds represent 90% of the essential oil. The qualitative chemical composition was the same. The

Table 2. Fungicidal Activity (MIC, Milligrams per Liter) of the Oils Compared with Synthetic Fungicides

	type A	type B	benomyl	tetraconazole	metalaxyl	chlozolinate	kresoxym-methyl
BOTCI	>1000	>1000				1.5	
CERBE	1000	500		0.6			
FUSXL	>1000	>1000	6.2				
FUSGR	>1000	500	0.6				
HELOR	>1000	1000		1.5			
PYTUL	250	62			0.1		
PYROR	>1000	500					25
RHISO	>1000	>1000	0.6				
SCLRO	125	62		1.5			
PHYCA	500	125			3.1		
SEPTR	1000	125	1.5				

Table 3. Major Compounds (Percent w/w) of the Essential Oil of *H. italicum* ssp. *microphyllum* in the Flowers and Stems for the Three Harvesting Periods

	June[a]	July[a]	Sept[a]
	Flowers		
yield % (v/p)	0.11 aA	0.13 abA	0.18 bA
limonene	5.9 aA	7.7 aA	1.5 bA
linalool	14.9 aA	22.9 bA	12.9 aA
α-terpineol	0.7 aA	0.7 aA	0.6 aA
nerol	8.6 aA	11.4 aA	12.0 aA
neryl acetate	26.6 aA	23.1 aA	16.6 bA
neryl propionate[b]	14.1 aA	9.7 bA	14.2 aA
γ-curcumene[b]	11.4 aA	6.0 bA	4.1 cA
ar-curcumene[b]	1.1 aA	2.6 aA	8.3 bA
guaiol	0.7 aA	1.0 aA	0.4 aA
rosifoliol[b]	6.2 aA	5.6 aA	18.2 bA
	Stems (Stem plus Leaves)		
yield % (v/p)	0.03 aB	0.03 aB	0.04 bB
limonene	17.8 aB	10.7 bA	2.4 cA
linalool	5.2 aB	5.9 aB	3.6 aB
α-terpineol	0.7 aA	0.7 aA	0.6 aA
nerol	9.6 aA	20.0 bB	9.5 aA
neryl acetate	21.5 aB	18.8 aB	23.9 aB
neryl propionate[b]	13.2 aA	11.9 aA	13.0 aA
γ-curcumene[b]	8.9 aA	6.1 aA	9.3 aA
ar-curcumene[b]	1.5 aA	3.4 aA	5.4 aA
guaiol	1.4 aA	1.0 aA	1.1 aA

[a] Lower case letters indicate comparison between harvesting periods. Capital letters indicate comparison between plant parts. [b] Expressed as nerol.

Table 4. Major Components (Percent w/w) of Essential Oil of *H. italicum* G. Don ssp. *microphyllum* (Willd) Nym in Four Samples from Distinct Plants

	sample[a]			
compound	1	2	3	4
limonene	4.5 a	4.9 b	2.5 c	9.0 d
linalool	7.4 a	15.2 b	26.5 c	8.0 a
α-terpineol	0.3 a	0.2 a	0.1 b	0.4 c
nerol	5.3 a	7.1 b	6.3 b	13.5 c
neryl acetate	35.7 a	26.4 b	20.0 b	27.2 a
neryl propionate[b]	5.2 a	10.2 b	1.9 c	15.6 d
γ-curcumene[b]	12.2 a	16.5 b	17.1 c	7.3 d
ar-curcumene[b]	4.5 a	4.8 a	6.8 b	4.0 a
guaiol	0.9 a	0.2 b	1.0 c	0.2 b
rosifoliol	15.1 a	6.5 b	6.8 c	5.8 b

[a] Lower case letters indicate comparison between the distinct plants. [b] Expressed as nerol.

quantitative composition was dependent on the period and the part of plant.

Differences Relating to the Harvesting Period. In flowers, nerol, α-terpineol, and guaiol were present irrespective of the harvesting period. Neryl acetate and limonene were the same in the first and second harvests and decreased in the third one. Rosifoliol and *ar*-curcumene were constant in the first and second harvests and increased in the third one. Neryl propionate showed the lowest value in the second harvest. γ-Curcumene decreased from the first to the third harvest, whereas linalool showed the highest value in the second harvest.

In the stems the content of limonene decreased from the first to the third harvest from 17.8 to 2.4%. Nerol increased during the flowering stage to 20% and decreased in the third harvest. Rosifoliol reached its highest value (20.1%) in the third one. All of the other compounds were independent of the harvesting period.

Differences Relating to the Parts of the Plant. At the first harvest (June) the major components of the flowers were linalool (14.9%), neryl acetate (26.6%), and neryl propionate (14,1%); the stems were richer in limonene (17.8% compared with 5.9% in flowers) and rosifoliol (12.1 and 6.2%, respectively); the concentrations of α-terpineol, nerol, neryl propionate, *ar*-curcumene, guaiol, and γ-curcumene were the same in the different parts of the plant. Neryl acetate was found at higher levels in the flowers (26.6 vs 21.5%).

At the second harvest (July) the concentration of linalool was still higher in the flowers by a factor of 4. Nerol and neryl acetate showed an opposite tendency, the former being more concentrated in the stems. Rosifoliol showed the same behavior. All of the other compounds were at similar concentrations in flowers and stems.

At the third harvest (September) the flowers were still richer in linalool, whereas the stems were richer than the flowers in neryl acetate (23.9 vs 16.6%). The concentrations of all other compounds in the flowers and stems were the same.

Influence of Sample Homogeneity. Because significant standard deviations were found in compound concentrations for the first samples (unpublished results), the distillation procedure as well as the analytical method was studied. Triplicate trials of the analytical and distillation procedures showed good reproducibility with standard deviations of <10%. The assumption that the diversity could be due to a considerable difference in the chemical composition of the plants was considered. **Table 4** shows the differences in the concentrations of the essential oil compounds, from distinct plants. Higher values were found for limonene 72.23% (between sample 3 and 4), linalool 72.08% (between samples 1 and 3), α-terpineol 75% (between samples 3 and 4), neryl propionate 87.83% (between samples 3 and 4), and guaiol 80% (between samples 3 and 4); lower values were found for *ar*-curcumene 41.18% (between samples 3 and 4), neryl acetate 43.98% (between samples 1 and 3), 57.31% γ-curcumene (between samples 3 and 4), 61.59% rosifoliol (between samples 1 and 4), and 60.74% nerol (between samples 1 and 4). Because laboratory analyses are carried out by using small amounts of samples, the large differences in concentration

of the single compounds could affect dramatically the quantitative composition of the essential oil.

Conclusions. The use of the RAPD technique to study the polymorphism among *Helichrysum* samples suggested the existence of two putative chemotypes, as shown by other chemical analyses. The objective definition of this suggestion will be achieved by analyzing more samples of *Helichrysum* using the same approach.

It is well-known that *Helichrysum* has a moderate antimicrobial activity, which is generally attributed to nerol esters (*3, 4*). These compounds are completely absent in the essential oil of *Helichrysum* type B, which unexpectedly showed a good antifungal action against *Pythium ultimum* and *Sclerotium rolfsii* and a moderate action against *Phytophthora capsici* and *Septoria tritici.*

The essential oil of type A is rich in nerol, and its esters, linalool and limonene. These compounds have nice perfume and for this reason are widely used in cosmetics. The concentration of these compounds is higher during the flowering stage and quite higher in the flowers than in the stems. Therefore, the distillation of flowers and stems together does not affect the commercial quality. Type A oils could be used in cosmetics and aromatherapy, whereas type B oils may possibly be employed as biopesticides

The homogenity of the sample to be analyzed in the laboratory is an important step to give correct quantitative information on the composition of the essential oil of an area or a cultivation.

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